

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
7 February 2002 (07.02.2002)

PCT

(10) International Publication Number
WO 02/10206 A2

(51) International Patent Classification⁷: **C07K 14/195**

(21) International Application Number: **PCT/EP01/08224**

(22) International Filing Date: **17 July 2001 (17.07.2001)**

(25) Filing Language: **English**

(26) Publication Language: **English**

(30) Priority Data:
100 53 942.4 2 August 2000 (02.08.2000) DE
101 09 686.0 28 February 2001 (28.02.2001) DE

(71) Applicant: **DEGUSSA AG** [DE/DE]; Bennigsenplatz 1,
40474 Düsseldorf (DE).

(72) Inventors: **BATHE, Brigitte**; Twieten 1, 33154 Salzkotten (DE). **MÖCKEL, Bettina**; Benrodestrasse 35, 40597

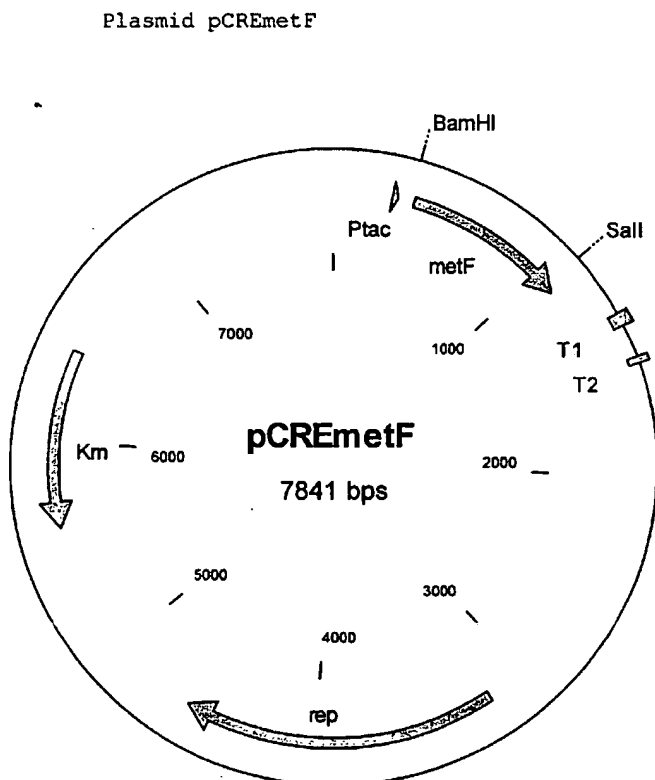
Düsseldorf (DE). **PFEFFERLE, Walter**; Jahnstrasse 33, 33790 Halle (Westf.) (DE). **HUTHMACHER, Klaus**; Lärchenweg 18, 63584 Gelnhausen (DE). **BINDER, Michael**; Kalberkamp 28, 33803 Steinhagen (Westf.) (DE). **GREISSINGER, Dieter**; Augasse 1f, 61194 Niddatal (DE). **THIERBACH, Georg**; Gunststrasse 21, 33613 Bielefeld (DE).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European

[Continued on next page]

(54) Title: **NUCLEOTIDE SEQUENCES WHICH CODE FOR THE METF GENE**



(57) Abstract: The invention relates to an isolated polynucleotide comprising a polynucleotide sequence chosen from the group consisting of a) polynucleotide which is identical to the extent of at least 70 % to a polynucleotide which codes for a polypeptide which comprises the amino acid sequence of SEQ ID No. 2, b) polynucleotide which codes for a polypeptide which comprises an amino acid sequence which is identical to the extent of at least 70% to the amino acid sequence of SEQ ID No. 2, c) polynucleotide which is complementary to the polynucleotides of a) or b), and d) polynucleotide comprising at least 15 successive nucleotides of the polynucleotide sequence of a), b) or c), and processes for the fermentative preparation of L-amino acids using coryneform bacteria in which at least the metF gene is present in enhanced form, and the use of the polynucleotide sequences as hybridization probes.



patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

— *with sequence listing part of description published separately in electronic form and available upon request from the International Bureau*

Published:

— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

Nucleotide sequences which code for the metF gene

Field of the Invention

The invention provides nucleotide sequences from coryneform bacteria which code for the metF gene and a process for the fermentative preparation of amino acids, in particular L-methionine, using bacteria in which the metF gene is enhanced.

Prior Art

L-Amino acids, in particular L-methionine, are used in human medicine and in the pharmaceuticals industry, in the foodstuffs industry and very particularly in animal nutrition.

It is known that amino acids are prepared by fermentation from strains of coryneform bacteria, in particular *Corynebacterium glutamicum*. Because of their great importance, work is constantly being undertaken to improve the preparation processes. Improvements to the process can relate to fermentation measures, such as, for example, stirring and supply of oxygen, or the composition of the nutrient media, such as, for example, the sugar concentration during the fermentation, or the working up to the product form by, for example, ion exchange chromatography, or the intrinsic output properties of the microorganism itself.

Methods of mutagenesis, selection and mutant selection are used to improve the output properties of these microorganisms. Strains which are resistant to antimetabolites, such as e.g. the methionine analogue α -methyl-methionine, ethionine, norleucine, N-acetylnorleucine, S-trifluoromethylhomocysteine, 2-amino-5-heprenoitic acid, seleno-methionine, methionine-sulfoximine, methoxine, 1-aminocyclopentane-carboxylic

acid, or are auxotrophic for metabolites of regulatory importance and produce amino acid, such as e.g. L-methionine, are obtained in this manner.

5 Methods of the recombinant DNA technique have also been employed for some years for improving the strain of *Corynebacterium* strains which produce L-amino acid, by amplifying individual amino acid biosynthesis genes and investigating the effect on the amino acid production.

Object of the Invention

10 The inventors had the object of providing new measures for improved fermentative preparation of amino acids, in particular L-methionine.

Summary of the Invention

15 When L-methionine or methionine are mentioned in the following, the salts, such as e.g. methionine hydrochloride or methionine sulfate are also meant by this.

The invention provides an isolated polynucleotide from coryneform bacteria, comprising a polynucleotide sequence which codes for the metF gene, chosen from the group
20 consisting of

- a) polynucleotide which is identical to the extent of at least 70% to a polynucleotide which codes for a polypeptide which comprises the amino acid sequence of SEQ ID No. 2,
- 25 b) polynucleotide which codes for a polypeptide which comprises an amino acid sequence which is identical to the extent of at least 70% to the amino acid sequence of SEQ ID No. 2,
- c) polynucleotide which is complementary to the
30 polynucleotides of a) or b), and

d) polynucleotide comprising at least 15 successive nucleotides of the polynucleotide sequence of a), b) or c),

the polypeptide preferably having the activity of methylene
5 tetrahydrofolate reductase.

The invention also provides the above-mentioned polynucleotide, this preferably being a DNA which is capable of replication, comprising:

- (i) the nucleotide sequence shown in SEQ ID No. 1, or
- 10 (ii) at least one sequence which corresponds to sequence (i) within the range of the degeneration of the genetic code, or
- (iii) at least one sequence which hybridizes with the sequence complementary to sequence (i) or (ii),
15 and optionally
- (iv) sense mutations of neutral function in (i).

The invention also provides

a polynucleotide comprising the nucleotide sequence as shown in SEQ ID No. 1,

20 a polynucleotide which codes for a polypeptide which comprises the amino acid sequence as shown in SEQ ID No. 2,

a vector containing the polynucleotide according to the invention, in particular a shuttle vector or plasmid
25 vector, and

and coryneform bacteria serving as the host cell, which contain the vector or in which the metF gene is enhanced.

The invention also provides polynucleotides which substantially comprise a polynucleotide sequence, which are obtainable by screening by means of hybridization of a corresponding gene library, which comprises the complete
5 gene with the polynucleotide sequence corresponding to SEQ ID No. 1, with a probe which comprises the sequence of the polynucleotide mentioned, according to SEQ ID No. 1 or a fragment thereof, and isolation of the DNA sequence mentioned.

10 Detailed Description of the Invention

Polynucleotides which comprise the sequences according to the invention are suitable as hybridization probes for RNA, cDNA and DNA, in order to isolate, in the full length,
15 nucleic acids or polynucleotides or genes which code for methylene tetrahydrofolate reductase or to isolate those nucleic acids or polynucleotides or genes which have a high similarity of sequence to methylene tetrahydrofolate reductase.

Polynucleotides which comprise the sequences according to
20 the invention are furthermore suitable as primers with the aid of which DNA of genes which code for methylene tetrahydrofolate reductase can be prepared by the polymerase chain reaction (PCR).

Such oligonucleotides which serve as probes or primers
25 comprise at least 30, preferably at least 20, very particularly preferably at least 15 successive nucleotides. Oligonucleotides which have a length of at least 40 or 50 nucleotides are also suitable. Oligonucleotides with a length of at least 100, 150, 200, 250 or 300 nucleotides
30 are optionally also suitable.

"Isolated" means separated out of its natural environment.

"Polynucleotide" in general relates to polyribonucleotides and polydeoxyribonucleotides, it being possible for these to be non-modified RNA or DNA or modified RNA or DNA.

5 "Polypeptides" are understood as meaning peptides or proteins which comprise two or more amino acids bonded via peptide bonds.

The polypeptides according to the invention include a polypeptide according to SEQ ID No. 2, in particular those with the biological activity of methylene tetrahydrofolate
10 reductase, and also those which are at least 70%, preferably at least 80% and in particular which are at least 90% to 95% identical to the polypeptide according to SEQ ID No. 2 and have the activity mentioned.

15 The invention moreover provides a process for the fermentative preparation of amino acids, in particular L-methionine, using coryneform bacteria which in particular already produce amino acids, and in which the nucleotide sequences which code for the metF gene are enhanced, in particular over-expressed.

20 The term "enhancement" in this connection describes the increase in the intracellular activity of one or more enzymes in a microorganism which are coded by the corresponding DNA, for example by increasing the number of copies of the gene or genes, using a potent promoter or
25 using a gene which codes for a corresponding enzyme having a high activity, and optionally combining these measures.

By enhancement measures, in particular over-expression, the activity or concentration of the corresponding protein is in general increased by at least 10%, 25%, 50%, 75%, 100%,
30 150%, 200%, 300%, 400% or 500%, up to a maximum of 1000% or 2000%, based on the starting microorganism.

The microorganisms which the present invention provides can prepare L-amino acids, in particular L-methionine, from

glucose, sucrose, lactose, fructose, maltose, molasses, starch, cellulose or from glycerol and ethanol. They can be representatives of coryneform bacteria, in particular of the genus *Corynebacterium*. Of the genus *Corynebacterium*, there may be mentioned in particular the species *Corynebacterium glutamicum*, which is known among experts for its ability to produce L-amino acids.

Suitable strains of the genus *Corynebacterium*, in particular of the species *Corynebacterium glutamicum* (*C. glutamicum*), are in particular the known wild-type strains

Corynebacterium glutamicum ATCC13032
Corynebacterium acetoglutamicum ATCC15806
Corynebacterium acetoacidophilum ATCC13870
Corynebacterium thermoaminogenes FERM BP-1539
Corynebacterium melassecola ATCC17965
Brevibacterium flavum ATCC14067
Brevibacterium lactofermentum ATCC13869 and
Brevibacterium divaricatum ATCC14020

or L-amino acid-producing mutants or strains prepared therefrom, such as, for example, the L-methionine-producing strain

Corynebacterium glutamicum ATCC21608.

The new *metF* gene from *C. glutamicum* which codes for the enzyme methylene tetrahydrofolate reductase [EC:1.7.99.5] has been isolated.

To isolate the *metF* gene or also other genes of *C. glutamicum*, a gene library of this microorganism is first set up in *Escherichia coli* (*E. coli*). The setting up of gene libraries is described in generally known textbooks and handbooks. The textbook by Winnacker: *Gene und Klone, Eine Einführung in die Gentechnologie* (Verlag Chemie, Weinheim, Germany, 1990), or the handbook by Sambrook et al.: *Molecular Cloning, A Laboratory Manual* (Cold Spring

Harbor Laboratory Press, 1989) may be mentioned as an example. A well-known gene library is that of the *E. coli* K-12 strain W3110 set up in λ vectors by Kohara et al. (Cell 50, 495-508 (1987)). Bathe et al. (Molecular and General Genetics, 252:255-265, 1996) describe a gene library of *C. glutamicum* ATCC13032, which was set up with the aid of the cosmid vector SuperCos I (Wahl et al., 1987, Proceedings of the National Academy of Sciences USA, 84:2160-2164) in the *E. coli* K-12 strain NM554 (Raleigh et al., 1988, Nucleic Acids Research 16:1563-1575).

Börmann et al. (Molecular Microbiology 6(3), 317-326 (1992)) in turn describe a gene library of *C. glutamicum* ATCC13032 using the cosmid pHCT9 (Hohn and Collins, Gene 11, 291-298 (1980)).

To prepare a gene library of *C. glutamicum* in *E. coli* it is also possible to use plasmids such as pBR322 (Bolivar, Life Sciences, 25, 807-818 (1979)) or pUC9 (Vieira et al., 1982, Gene, 19:259-268). Suitable hosts are, in particular, those *E. coli* strains which are restriction- and recombination-defective. An example of these is the strain DH5 α mc r , which has been described by Grant et al. (Proceedings of the National Academy of Sciences USA, 87 (1990) 4645-4649). The long DNA fragments cloned with the aid of cosmids can in turn be subcloned in the usual vectors suitable for sequencing and then sequenced, as is described e.g. by Sanger et al. (Proceedings of the National Academy of Sciences of the United States of America, 74:5463-5467, 1977).

The resulting DNA sequences can then be investigated with known algorithms or sequence analysis programs, such as e.g. that of Staden (Nucleic Acids Research 14, 217-232(1986)), that of Marck (Nucleic Acids Research 16, 1829-1836 (1988)) or the GCG program of Butler (Methods of Biochemical Analysis 39, 74-97 (1998)).

The new DNA sequence of *C. glutamicum* which codes for the metF gene and which, as SEQ ID No. 1, is a constituent of the present invention has been found. The amino acid sequence of the corresponding protein has furthermore been
5 derived from the present DNA sequence by the methods described above. The resulting amino acid sequence of the metF gene product is shown in SEQ ID No. 2.

Coding DNA sequences which result from SEQ ID No. 1 by the degeneracy of the genetic code are also a constituent of
10 the invention. In the same way, DNA sequences which hybridize with SEQ ID No. 1 or parts of SEQ ID No. 1 are a constituent of the invention. Conservative amino acid exchanges, such as e.g. exchange of glycine for alanine or of aspartic acid for glutamic acid in proteins, are
15 furthermore known among experts as "sense mutations" which do not lead to a fundamental change in the activity of the protein, i.e. are of neutral function.

It is furthermore known that changes on the N and/or C terminus of a protein cannot substantially impair or can
20 even stabilize the function thereof. Information in this context can be found by the expert, inter alia, in Ben-Bassat et al. (Journal of Bacteriology 169:751-757 (1987)), in O'Regan et al. (Gene 77:237-251 (1989)), in Sahin-Toth et al. (Protein Sciences 3:240-247 (1994)), in Hochuli et
25 al. (Bio/Technology 6:1321-1325 (1988)) and in known textbooks of genetics and molecular biology. Amino acid sequences which result in a corresponding manner from SEQ ID No. 2 are also a constituent of the invention.

In the same way, DNA sequences which hybridize with SEQ ID
30 No. 1 or parts of SEQ ID No. 1 are a constituent of the invention. Finally, DNA sequences which are prepared by the polymerase chain reaction (PCR) using primers which result from SEQ ID No. 1 are a constituent of the invention. Such oligonucleotides typically have a length of at least 15
35 nucleotides.

Instructions for identifying DNA sequences by means of hybridization can be found by the expert, inter alia, in the handbook "The DIG System Users Guide for Filter Hybridization" from Boehringer Mannheim GmbH (Mannheim, Germany, 1993) and in Liebl et al. (International Journal of Systematic Bacteriology (1991) 41: 255-260).

Instructions for amplification of DNA sequences with the aid of the polymerase chain reaction (PCR) can be found by the expert, inter alia, in the handbook by Gait: Oligonucleotide Synthesis: A Practical Approach (IRL Press, Oxford, UK, 1984) and in Newton and Graham: PCR (Spektrum Akademischer Verlag, Heidelberg, Germany, 1994).

It has been found that coryneform bacteria produce amino acids, in particular L-methionine, in an improved manner after over-expression of the metF gene.

To achieve an over-expression, the number of copies of the corresponding genes can be increased, or the promoter and regulation region or the ribosome binding site upstream of the structural gene can be mutated. Expression cassettes which are incorporated upstream of the structural gene act in the same way. By inducible promoters, it is additionally possible to increase the expression in the course of fermentative L-methionine production. The expression is likewise improved by measures to prolong the life of the m-RNA. Furthermore, the enzyme activity is also increased by preventing the degradation of the enzyme protein. The genes or gene constructs can either be present in plasmids with a varying number of copies, or can be integrated and amplified in the chromosome. Alternatively, an over-expression of the genes in question can furthermore be achieved by changing the composition of the media and the culture procedure.

Instructions in this context can be found by the expert, inter alia, in Martin et al. (Bio/Technology 5, 137-146 (1987)), in Guerrero et al. (Gene 138, 35-41 (1994)),

Tsuchiya and Morinaga (Bio/Technology 6, 428-430 (1988)),
in Eikmanns et al. (Gene 102, 93-98 (1991)), in European
Patent Specification 0 472 869, in US Patent 4,601,893, in
Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991)), in
5 Reinscheid et al. (Applied and Environmental Microbiology
60, 126-132 (1994)), in LaBarre et al. (Journal of
Bacteriology 175, 1001-1007 (1993)), in Patent Application
WO 96/15246, in Malumbres et al. (Gene 134, 15 - 24
(1993)), in Japanese Laid-Open Specification
10 JP-A-10-229891, in Jensen and Hammer (Biotechnology and
Bioengineering 58, 191-195 (1998)), in Makrides
(Microbiological Reviews 60:512-538 (1996)) and in known
textbooks of genetics and molecular biology.

By way of example, for enhancement the metF gene according
15 to the invention was over-expressed with the aid of
episomal plasmids. Suitable plasmids are those which are
replicated in coryneform bacteria. Numerous known plasmid
vectors, such as e.g. pZ1 (Menkel et al., Applied and
Environmental Microbiology (1989) 64: 549-554), pEKEx1
20 (Eikmanns et al., Gene 102:93-98 (1991)) or pHS2-1 (Sonnen
et al., Gene 107:69-74 (1991)) are based on the cryptic
plasmids pHM1519, pBL1 or pGA1. Other plasmid vectors, such
as e.g. those based on pCG4 (US-A 4,489,160), or pNG2
(Serwold-Davis et al., FEMS Microbiology Letters 66, 119-
25 124 (1990)), or pAG1 (US-A 5,158,891), can be used in the
same manner.

Plasmid vectors which are furthermore suitable are also
those with the aid of which the process of gene
amplification by integration into the chromosome can be
30 used, as has been described, for example, by Reinscheid et
al. (Applied and Environmental Microbiology 60, 126-132
(1994)) for duplication or amplification of the hom-thrB
operon. In this method, the complete gene is cloned in a
plasmid vector which can replicate in a host (typically E.
35 coli), but not in C. glutamicum. Possible vectors are, for

example, pSUP301 (Simon et al., Bio/Technology 1, 784-791 (1983)), pK18mob or pK19mob (Schäfer et al., Gene 145, 69-73 (1994)), pGEM-T (Promega corporation, Madison, WI, USA), pCR2.1-TOPO (Shuman (1994). Journal of Biological Chemistry 269:32678-84; US-A 5,487,993), pCR@Blunt (Invitrogen, Groningen, Holland; Bernard et al., Journal of Molecular Biology, 234: 534-541 (1993)), pEM1 (Schrumpf et al, 1991, Journal of Bacteriology 173:4510-4516) or pBGS8 (Spratt et al., 1986, Gene 41: 337-342). The plasmid vector which contains the gene to be amplified is then transferred into the desired strain of *C. glutamicum* by conjugation or transformation. The method of conjugation is described, for example, by Schäfer et al. (Applied and Environmental Microbiology 60, 756-759 (1994)). Methods for transformation are described, for example, by Thierbach et al. (Applied Microbiology and Biotechnology 29, 356-362 (1988)), Dunican and Shivanan (Bio/Technology 7, 1067-1070 (1989)) and Tauch et al. (FEMS Microbiological Letters 123, 343-347 (1994)). After homologous recombination by means of a "cross over" event, the resulting strain contains at least two copies of the gene in question.

In addition, it may be advantageous for the production of amino acids, in particular L-methionine, to enhance one or more enzymes of the particular biosynthesis pathway, of glycolysis, of anaplerosis, of the citric acid cycle or of amino acid export, in addition to the metF gene.

Thus for the preparation of amino acids, in particular L-methionine, one or more genes chosen from the group consisting of

- the gap gene which codes for glyceraldehyde 3-phosphate dehydrogenase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- the tpi gene which codes for triose phosphate isomerase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),

- the pgk gene which codes for 3-phosphoglycerate kinase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- the pyc gene which codes for pyruvate carboxylase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- 5 • the lysC gene which codes for a feed-back resistant aspartate kinase (ACCESSION NUMBER P26512 ; EP-B-0387527; EP-A-0699759),
- the metA gene which codes for homoserine O-acetyltransferase (ACCESSION Number AF052652),
- 10 • the metB gene which codes for cystathionine gamma-synthase (ACCESSION Number AF126953),
- the aecD gene which codes for cystathionine gamma-lyase (ACCESSION Number M89931)
- the glyA gene which codes for serine
- 15 hydroxymethyltransferase (JP-A-08107788),
- the metY gene which codes for O-acetylhomoserine sulfhydrylase (DSM 13556)

can be enhanced, in particular over-expressed.

20 It may furthermore be advantageous for the production of amino acids, in particular L-methionine, in addition to the enhancement of the metF gene, for one or more genes chosen from the group consisting of

- the thrB gene which codes for homoserine kinase (ACCESSION Number P08210),
- 25 • the ilvA gene which codes for threonine dehydratase (ACCESSION Number Q04513),
- the thrC gene which codes for threonine synthase (ACCESSION Number P23669),

- the ddh gene which codes for meso-diaminopimelate D-dehydrogenase (ACCESSION Number Y00151),
- the pck gene which codes for phosphoenol pyruvate carboxykinase (DE 199 50 409.1; DSM 13047),
- 5 • the pgi gene which codes for glucose 6-phosphate isomerase (US 09/396,478; DSM 12969),
- the poxB gene which codes for pyruvate oxidase (DE: 1995 1975.7; DSM 13114)

10 to be attenuated, in particular for the expression thereof to be reduced.

The term "attenuation" in this connection describes the reduction or elimination of the intracellular activity of one or more enzymes (proteins) in a microorganism which are coded by the corresponding DNA, for example by using a weak
15 promoter or using a gene or allele which codes for a corresponding enzyme with a low activity or inactivates the corresponding gene or enzyme (protein), and optionally combining these measures.

By attenuation measures, the activity or concentration of
20 the corresponding protein is in general reduced to 0 to 50%, 0 to 25%, 0 to 10% or 0 to 5% of the activity or concentration of the wild-type protein.

In addition to over-expression of the metF gene it may furthermore be advantageous, for the production of amino
25 acids, in particular L-methionine, to eliminate undesirable side reactions, (Nakayama: "Breeding of Amino Acid Producing Micro-organisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

30 The microorganisms prepared according to the invention can be cultured continuously or discontinuously in the batch

- process (batch culture) or in the fed batch (feed process) or repeated fed batch process (repetitive feed process) for the purpose of production of amino acids, in particular L-methionine. A summary of known culture methods is described
- 5 in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).
- 10 The culture medium to be used must meet the requirements of the particular strains in a suitable manner. Descriptions of culture media for various microorganisms are contained in the handbook "Manual of Methods for General
- 15 Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981).
- Sugars and carbohydrates, such as e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and cellulose, oils and fats, such as e.g. soya oil, sunflower oil, groundnut oil and coconut fat, fatty acids, such as e.g.
- 20 palmitic acid, stearic acid and linoleic acid, alcohols, such as e.g. glycerol and ethanol, and organic acids, such as e.g. acetic acid, can be used as the source of carbon. These substance can be used individually or as a mixture.
- Organic nitrogen-containing compounds, such as peptones,
- 25 yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic compounds, such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate, can be used as the source of nitrogen. The sources of nitrogen can
- 30 be used individually or as a mixture.
- Organic and inorganic sulfur-containing compounds, such as, for example, sulfides, sulfites, sulfates and thiosulfates, can be used as a source of sulfur, in particular for the preparation of methionine.

Phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts can be used as the source of phosphorus. The culture medium must furthermore comprise salts of
5 metals, such as e. g. magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, can be employed in addition to the above-mentioned substances. Suitable precursors can moreover be added to the culture
10 medium. The starting substances mentioned can be added to the culture in the form of a single batch, or can be fed in during the culture in a suitable manner.

Basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia or aqueous ammonia, or acid compounds,
15 such as phosphoric acid or sulfuric acid, can be employed in a suitable manner to control the pH of the culture. Antifoams, such as e.g. fatty acid polyglycol esters, can be employed to control the development of foam. Suitable substances having a selective action, such as e.g.
20 antibiotics, can be added to the medium to maintain the stability of plasmids. To maintain aerobic conditions, oxygen or oxygen-containing gas mixtures, such as e.g. air, are introduced into the culture. The temperature of the culture is usually 20°C to 45°C, and preferably 25°C to
25 40°C. Culturing is continued until a maximum of the desired product has formed. This target is usually reached within 10 hours to 160 hours.

The fermentation broths obtained in this way, in particular containing L-methionine, usually have a dry weight of 7.5
30 to 25 wt.% and contain L-methionine. It is furthermore also advantageous if the fermentation is conducted in a sugar-limited procedure at least at the end, but in particular over at least 30% of the duration of the fermentation. That is to say, the concentration of utilizable sugar in the

fermentation medium is reduced to ≥ 0 to 3 g/l during this period.

The fermentation broth prepared in this manner, in particular containing L-methionine, is then further
5 processed. Depending on requirements, all or some of the biomass can be removed from the fermentation broth by separation methods, such as e.g. centrifugation, filtration, decanting or a combination thereof, or it can be left completely in this. This broth is then thickened or
10 concentrated by known methods, such as e.g. with the aid of a rotary evaporator, thin film evaporator, falling film evaporator, by reverse osmosis, or by nanofiltration. This concentrated fermentation broth can then be worked up by methods of freeze drying, spray drying, spray granulation
15 or by other processes to give a preferably free-flowing, finely divided powder.

This free-flowing, finely divided powder can then in turn be converted by suitable compacting or granulating processes into a coarse-grained, readily free-flowing,
20 storable and largely dust-free product. In the granulation or compacting it is advantageous to employ conventional organic or inorganic auxiliary substances or carriers, such as starch, gelatin, cellulose derivatives or similar substances, such as are conventionally used as binders,
25 gelling agents or thickeners in foodstuffs or feedstuffs processing, or further substances, such as, for example, silicas, silicates or stearates.

"Free-flowing" is understood as meaning powders which flow unimpeded out of the vessel with the opening of 5 mm
30 (millimeters) of a series of glass outflow vessels with outflow openings of various sizes (Klein, Seifen, Öle, Fette, Wachse 94, 12 (1968)).

As described here, "finely divided" means a powder with a predominant content ($> 50\%$) with a particle size of 20 to

200 μm diameter. "Coarse-grained" means products with a predominant content ($> 50\%$) with a particle size of 200 to 2000 μm diameter. In this context, "dust-free" means that the product contains only small contents ($< 5\%$) with particle sizes of less than 20 μm diameter. The particle size determination can be carried out with methods of laser diffraction spectrometry. The corresponding methods are described in the textbook on "Teilchengrößenmessung in der Laborpraxis" by R. H. Müller and R. Schuhmann, Wissenschaftliche Verlagsgesellschaft Stuttgart (1996) or in the textbook "Introduction to Particle Technology" by M. Rhodes, Verlag Wiley & Sons (1998).

"Storable" in the context of this invention means a product which can be stored for up to 120 days, preferably up to 52 weeks, particularly preferably 60 months, without a substantial loss ($< 5\%$) of methionine occurring.

Alternatively, however, the product can be absorbed on to an organic or inorganic carrier substance which is known and conventional in feedstuffs processing, such as, for example, silicas, silicates, grits, brans, meals, starches, sugars or others, and/or mixed and stabilized with conventional thickeners or binders. Use examples and processes in this context are described in the literature (Die Mühle + Mischfuttertechnik 132 (1995) 49, page 817).

Finally, the product can be brought into a state in which it is stable to digestion by animal stomachs, in particular the stomach of ruminants, by coating processes ("coating") using film-forming agents, such as, for example, metal carbonates, silicas, silicates, alginates, stearates, starches, gums and cellulose ethers, as described in DE-C-4100920.

If the biomass is separated off during the process, further inorganic solids, for example added during the fermentation, are in general removed. In addition, the

animal feedstuffs additive according to the invention comprises at least the predominant proportion of the further substances, in particular organic substances, which are formed or added and are present in solution in the
5 fermentation broth, where these have not been separated off by suitable processes.

In one aspect of the invention, the biomass can be separated off to the extent of up to 70%, preferably up to 80%, preferably up to 90%, preferably up to 95%, and
10 particularly preferably up to 100%. In another aspect of the invention, up to 20% of the biomass, preferably up to 15%, preferably up to 10%, preferably up to 5%, particularly preferably no biomass is separated off.

These organic substances include organic by-products which
15 are optionally produced, in addition to the L-methionine, and optionally discharged by the microorganisms employed in the fermentation. These include L-amino acids chosen from the group consisting of L-lysine, L-valine, L-threonine, L-alanine or L-tryptophan. They include vitamins chosen from
20 the group consisting of vitamin B1 (thiamine), vitamin B2 (riboflavin), vitamin B5 (pantothenic acid), vitamin B6 (pyridoxine), vitamin B12 (cyanocobalamin), nicotinic acid/nicotinamide and vitamin E (tocopherol). They include furthermore organic acids which carry one to three carboxyl
25 groups, such as, for example, acetic acid, lactic acid, citric acid, malic acid or fumaric acid. Finally, they also include sugars, such as, for example, trehalose. These compounds are optionally desired if they improve the nutritional value of the product.

30 These organic substances, including L-methionine and/or D-methionine and/or the racemic mixture D,L-methionine, can also be added, depending on requirements, as a concentrate or pure substance in solid or liquid form during a suitable process step. These organic substances mentioned can be
35 added individually or as mixtures to the resulting or

concentrated fermentation broth, or also during the drying or granulation process. It is likewise possible to add an organic substance or a mixture of several organic substances to the fermentation broth and a further organic substance or a further mixture of several organic substances during a later process step, for example granulation.

--The product described above is suitable as a feedstuffs additive, i.e. feed additive, for animal nutrition.

10 The L-methionine content of the animal feedstuffs additive is conventionally 1 wt.% to 80 wt.%, preferably 2 wt.% to 80 wt.%, particularly preferably 4 wt.% to 80 wt.%, and very particularly preferably 8 wt.% to 80 wt.%, based on the dry weight of the animal feedstuffs additive. Contents
15 of 1 wt.% to 60 wt.%, 2 wt.% to 60 wt.%, 4 wt.% to 60 wt.%, 6 wt.% to 60 wt.%, 1 wt.% to 40 wt.%, 2 wt.% to 40 wt.% or 4 wt.% to 40 wt.% are likewise possible. The water content of the feedstuffs additive is conventionally up to 5 wt.%, preferably up to 4 wt.%, and particularly preferably less
20 than 2 wt.%.

The invention accordingly also provides a process for the preparation of an L-methionine-containing animal feedstuffs additive from fermentation broths, which comprises the steps

- 25 a) culture and fermentation of an L-methionine-producing microorganism in a fermentation medium;
- b) removal of water from the L-methionine-containing fermentation broth (concentration);
- c) removal of an amount of 0 to 100 wt.% of the biomass
30 formed during the fermentation; and

- d) drying of the fermentation broth obtained according to a) and/or b) to obtain the animal feedstuffs additive in the desired powder or granule form.

If desired, one or more of the following steps can
5 furthermore be carried out in the process according to the invention:

- e) addition of one or more organic substances, including L-methionine and/or D-methionine and/or the racemic mixture D,L-methionine, to the products obtained
10 according to a), b) and/or c);
- f) addition of auxiliary substances chosen from the group consisting of silicas, silicates, stearates, grits and bran to the substances obtained according to a) to d) for stabilization and to increase the storability; or
- 15 g) conversion of the substances obtained according to a) to e) into a form stable to the animal stomach, in particular rumen, by coating with film-forming agents.

The analysis of L-methionine can be carried out by ion exchange chromatography with subsequent ninhydrin
20 derivation, as described by Spackman et al. (Analytical Chemistry, 30, (1958), 1190).

The process according to the invention is used for the fermentative preparation of amino acids, in particular L-methionine.

25 The present invention is explained in more detail in the following with the aid of embodiment examples.

Example 1

Preparation of a genomic cosmid gene library from
Corynebacterium glutamicum ATCC 13032

- Chromosomal DNA from *Corynebacterium glutamicum* ATCC 13032
5 was isolated as described by Tauch et al. (1995, Plasmid
33:168-179) and partly cleaved with the restriction enzyme
Sau3AI (Amersham Pharmacia, Freiburg, Germany, Product
Description Sau3AI, Code no. 27-0913-02). The DNA fragments
were dephosphorylated with shrimp alkaline phosphatase
10 (Roche Diagnostics GmbH, Mannheim, Germany, Product
Description SAP, Code no. 1758250). The DNA of the cosmid
vector SuperCos1 (Wahl et al. (1987) Proceedings of the
National Academy of Sciences USA 84:2160-2164), obtained
from Stratagene (La Jolla, USA, Product Description
15 SuperCos1 Cosmid Vector Kit, Code no. 251301) was cleaved
with the restriction enzyme XbaI (Amersham Pharmacia,
Freiburg, Germany, Product Description XbaI, Code no. 27-
0948-02) and likewise dephosphorylated with shrimp alkaline
phosphatase.
- 20 The cosmid DNA was then cleaved with the restriction enzyme
BamHI (Amersham Pharmacia, Freiburg, Germany, Product
Description BamHI, Code no. 27-0868-04). The cosmid DNA
treated in this manner was mixed with the treated ATCC13032
DNA and the batch was treated with T4 DNA ligase (Amersham
25 Pharmacia, Freiburg, Germany, Product Description T4-DNA-
Ligase, Code no. 27-0870-04). The ligation mixture was then
packed in phages with the aid of Gigapack II XL Packing
Extract (Stratagene, La Jolla, USA, Product Description
Gigapack II XL Packing Extract, Code no. 200217).
- 30 For infection of the *E. coli* strain NM554 (Raleigh et al.
1988, Nucleic Acid Research 16:1563-1575) the cells were
taken up in 10 mM MgSO₄ and mixed with an aliquot of the
phage suspension. The infection and titering of the cosmid
library were carried out as described by Sambrook et al.

(1989, Molecular Cloning: A laboratory Manual, Cold Spring Harbor), the cells being plated out on LB agar (Lennox, 1955, Virology, 1:190) with 100 mg/l ampicillin. After incubation overnight at 37°C, recombinant individual clones
5 were selected.

Example 2

Isolation and sequencing of the metF gene

The cosmid DNA of an individual colony was isolated with the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and partly cleaved with the restriction enzyme
10 Sau3AI (Amersham Pharmacia, Freiburg, Germany, Product Description Sau3AI, Product No. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline
15 phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, Product Description SAP, Product No. 1758250). After separation by gel electrophoresis, the cosmid fragments in the size range of 1500 to 2000 bp were isolated with the QiaExII Gel Extraction Kit (Product No. 20021, Qiagen,
20 Hilden, Germany).

The DNA of the sequencing vector pZero-1, obtained from Invitrogen (Groningen, The Netherlands, Product Description Zero Background Cloning Kit, Product No. K2500-01) was cleaved with the restriction enzyme BamHI (Amersham
25 Pharmacia, Freiburg, Germany, Product Description BamHI, Product No. 27-0868-04). The ligation of the cosmid fragments in the sequencing vector pZero-1 was carried out as described by Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor), the DNA mixture
30 being incubated overnight with T4 ligase (Pharmacia Biotech, Freiburg, Germany). This ligation mixture was then electroporated (Tauch et al. 1994, FEMS Microbiol Letters, 123:343-7) into the E. coli strain DH5 α MCR (Grant, 1990, Proceedings of the National Academy of Sciences U.S.A.,

87:4645-4649) and plated out on LB agar (Lennox, 1955, Virology, 1:190) with 50 mg/l zeocin.

- The plasmid preparation of the recombinant clones was carried out with Biorobot 9600 (Product No. 900200, Qiagen, Hilden, Germany). The sequencing was carried out by the dideoxy chain termination method of Sanger et al. (1977, Proceedings of the National Academy of Sciences U.S.A., 74:5463-5467) with modifications according to Zimmermann et al. (1990, Nucleic Acids Research, 18:1067). The "RR dRhodamin Terminator Cycle Sequencing Kit" from PE Applied Biosystems (Product No. 403044, Weiterstadt, Germany) was used. The separation by gel electrophoresis and analysis of the sequencing reaction were carried out in a "Rotiphoresis NF Acrylamide/Bisacrylamide" Gel (29:1) (Product No. A124.1, Roth, Karlsruhe, Germany) with the "ABI Prism 377" sequencer from PE Applied Biosystems (Weiterstadt, Germany).

- The raw sequence data obtained were then processed using the Staden program package (1986, Nucleic Acids Research, 14:217-231) version 97-0. The individual sequences of the pZerol derivatives were assembled to a continuous contig. The computer-assisted coding region analysis was prepared with the XNIP program (Staden, 1986, Nucleic Acids Research, 14:217-231).

- The resulting nucleotide sequence is shown in SEQ ID No. 1. Analysis of the nucleotide sequence showed an open reading frame of 1046 base pairs, which was called the metF gene. The metF gene codes for a protein of 349 amino acids.

Example 3

Preparation of the strain *C. glutamicum* ATCC13032/pCREmetF

3.1 Amplification of the metF gene

From the strain ATCC13032, chromosomal DNA was isolated by
5 the method of Eikmanns et al. (Microbiology 140: 1817 -1828
(1994)). Starting from the nucleotide sequences of the
methionine biosynthesis genes metF (SEQ ID No. 1) of *C.*
glutamicum ATCC13032, the following oligonucleotides were
chosen for the polymerase chain reaction (PCR) (see SEQ ID
10 No. 3 and SEQ ID No. 4):

metF-EVP5:

5'-GATCTAGGATCCAAAGGAGGACAACCATGTCCCTAACGAACATCCC-3'

metF-EVP3:

5'-GATCTACTCGAGTTCTTCTAGTTGGCTCGGCA-3'

15 The primers shown were synthesized by MWG-Biotech AG
(Ebersberg, Germany) and the PCR reaction was carried out
by the standard PCR method of Innis et al. (PCR protocols.
A guide to methods and applications, 1990, Academic Press)
with Pwo-Polymerase from Roche Diagnostics GmbH (Mannheim,
20 Germany). With the aid of the polymerase chain reaction,
the primers allow amplification of a DNA fragment 792 bp in
size, which carries the complete metF gene, which is
suitable for expression.

Furthermore, the primer metF-EVP5 contains the sequence for
25 the cleavage site of the restriction endonuclease BamHI and
the primer metF-EVP3 the cleavage site of the restriction
endonuclease XhoI, which are marked by underlining in the
nucleotide sequence shown above.

The metF fragment 792 bp in size was cleaved with the
30 restriction endonucleases BamHI and XhoI. The batch was
separated by gel electrophoresis and the metF fragment was

then isolated from the agarose gel with the QiaExII Gel Extraction Kit (Product No. 20021, Qiagen, Hilden, Germany).

3.2 Cloning of metF in the vector pZ8-1

- 5 The *E. coli* - *C. glutamicum* shuttle expression vector pZ8-1 (EP 0 375 889) was used as the base vector for the expression.

- DNA of the plasmid pZ8-1 was cleaved completely with the restriction enzymes BamHI and SalI and then
10 dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, Product Description SAP, Product No. 1758250).

- The metF fragment isolated from the agarose gel in example 3.1 and cleaved with the restriction endonucleases BamHI
15 and XhoI was mixed with the vector pZ8-1 prepared in this way and the batch was treated with T4 DNA ligase (Amersham Pharmacia, Freiburg, Germany, Product Description T4-DNA-Ligase, Code no.27-0870-04).

- The ligation batch was transformed in the *E. coli* strain
20 DH5 α mc^r (Hanahan, In: DNA cloning. A Practical Approach. Vol. I. IRL-Press, Oxford, Washington DC, USA). Selection of plasmid-carrying cells was made by plating out the transformation batch on LB agar (Lennox, 1955, Virology, 1:190) with 50 mg/l kanamycin. After incubation overnight
25 at 37°C, recombinant individual clones were selected. Plasmid DNA was isolated from a transformant with the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and checked by restriction cleavage. The
30 resulting plasmid was called pCREmetF.

3.3 Preparation of the strain *C. glutamicum* ATCC13032/pCREmetF

The vector pCREmetF obtained in example 3.2 was electroporated in the strain *C. glutamicum* ATCC13032 using the electroporation method described by Liebl et al. (FEMS Microbiology Letters, 53:299-303 (1989)). Selection of the plasmid-carrying cells took place on LBHIS agar comprising 18.5 g/l brain-heart infusion broth, 0.5 M sorbitol, 5 g/l Bacto-tryptone, 2.5 g/l Bacto-yeast extract, 5 g/l NaCl and 18 g/l Bacto-agar, which had been supplemented with 25 mg/l kanamycin. Incubation was carried out for 2 days at 33°C.

Plasmid DNA was isolated from a transformant by conventional methods (Peters-Wendisch et al., 1998, Microbiology 144, 915-927) and checked by restriction cleavage. The resulting strain was called ATCC13032pCREmetF.

Example 4

Preparation of methionine with the strain *C. glutamicum* ATCC13032/pCREmetF

The *C. glutamicum* strain ATCC13032/pCREmetF obtained in example 3 was cultured in a nutrient medium suitable for the production of methionine and the methionine content in the culture supernatant was determined.

For this, the strain was first incubated on an agar plate with the corresponding antibiotic (brain-heart agar with kanamycin (25 mg/l)) for 24 hours at 33°C. Starting from this agar plate culture, a preculture was seeded (10 ml medium in a 100 ml conical flask). The medium MM was used as the medium for the preculture.

Medium MM

CSL (corn steep liquor)	5 g/l
MOPS (morpholinopropanesulfonic acid)	20 g/l
Glucose (autoclaved separately)	50g/l
Salts:	
(NH ₄) ₂ SO ₄	25 g/l
KH ₂ PO ₄	0.1 g/l
MgSO ₄ * 7 H ₂ O	1.0 g/l
CaCl ₂ * 2 H ₂ O	10 mg/l
FeSO ₄ * 7 H ₂ O	10 mg/l
MnSO ₄ * H ₂ O	5.0mg/l
Biotin (sterile-filtered)	0.01 mg/l
Vitamin B12 (sterile-filtered)	0.02 mg/l
Thiamine * HCl (sterile-filtered)	0.2 mg/l
CaCO ₃	25 g/l

The CSL, MOPS and the salt solution were brought to pH 7 with aqueous ammonia and autoclaved. The sterile substrate and vitamin solutions were then added, as well as the CaCO₃ autoclaved in the dry state.

Kanamycin (25 mg/l) was added to this. The preculture was incubated for 16 hours at 33°C at 240 rpm on a shaking machine. A main culture was seeded from this preculture such that the initial OD (660 nm) of the main culture was 0.1. Medium MM was also used for the main culture.

Culturing is carried out in a 10 ml volume in a 100 ml conical flask with baffles. Kanamycin (25 mg/l) was added. Culturing was carried out at 33°C and 80% atmospheric humidity.

- 5 After 72 hours, the OD was determined at a measurement wavelength of 660 nm with a Biomek 1000 (Beckmann Instruments GmbH, Munich). The amount of methionine formed was determined with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column derivation with ninhydrin detection.
- 10

The result of the experiment is shown in Table 1.

Table 1

Strain	OD (660 nm)	Methionine mg/l
ATCC13032	10.3	1.4
ATCC13032/pCREmetF	11.2	7.3

Brief Description of the Figure:

- 15 • Figure 1: Plasmid pCREmetF

The abbreviations used have the following meaning:

- Km: Resistance gene for kanamycin
- metF: metF gene of *C. glutamicum*
- Ptac: tac promoter
- 20 T1 T2: Terminator T1T2 of the *rrnB* gene of *E. coli*
- rep: Plasmid-coded replication origin for *C. glutamicum* (of pHM1519)

BamHI: Cleavage site of the restriction enzyme BamHI

SalI: Cleavage site of the restriction enzyme SalI

SEQUENCE PROTOCOL

<110> Degussa AG

5 <120> Nucleotide sequences which code for the metF gene

<130> 000363 BT

<140>

10 <141>

<160> 4

<170> PatentIn Ver. 2.1

15

<210> 1

<211> 1551

<212> DNA

<213> Corynebacterium glutamicum

20

<220>

<221> CDS

<222> (299)..(1345)

<223> metF gene

25

<400> 1

gcgtcaagga cggactcaag tttttcagaa gaattcttat ggccttgccg cgccaggaaa 60

ccagcccacg cataaagagg acggattcgc tttcctccat tgagcacgaa actgcgaaga 120

30

tgggccacag catctgtgac aggagcgccg atatcagcaa ttgttagctc ttgagcatcg 180

aggaactgcg tcaaacgata tcgcacgacc tccggaaatt tgtcgaggtc aaggtcatgg 240

35

gcatcgaaac tgctcaagga gacgtccttc aatcgaatag ggggatgccc gctgaatt 298

ttg gtg gag gtg aat aaa tgc cag agg cag tcc caa caa aac act ctc 346

Met Val Glu Val Asn Lys Cys Gln Arg Gln Ser Gln Gln Asn Thr Leu

40

1 5 10 15

atc aca cta aga tac cca ggc atg tcc cta acg aac atc cca gcc tca 394

Ile Thr Leu Arg Tyr Pro Gly Met Ser Leu Thr Asn Ile Pro Ala Ser

20 25 30

45

tct caa tgg gca att agc gac gtt ttg aag cgt cct tca ccc ggc cga 442

Ser Gln Trp Ala Ile Ser Asp Val Leu Lys Arg Pro Ser Pro Gly Arg

35 40 45

50

gta cct ttt tct gtc gag ttt atg cca ccc cgc gac gat gca gct gaa 490

Val Pro Phe Ser Val Glu Phe Met Pro Pro Arg Asp Asp Ala Ala Glu

50 55 60

55

gag cgt ctt tac cgc gca gca gag gtc ttc cat gac ctc ggt gca tcg 538

Glu Arg Leu Tyr Arg Ala Ala Glu Val Phe His Asp Leu Gly Ala Ser

65 70 75 80

ttt gtc tcc gtg act tat ggt gct ggc gga tca acc cgt gag aga acc 586

Phe Val Ser Val Thr Tyr Gly Ala Gly Gly Ser Thr Arg Glu Arg Thr

85 90 95

5	tca cgt att gct cga cga tta gcg aaa caa ccg ttg acc act ctg gtg	634
	Ser Arg Ile Ala Arg Arg Leu Ala Lys Gln Pro Leu Thr Thr Leu Val	
	100 105 110	
10	cac ctg acc ctg gtt aac cac act cgc gaa gag atg aag gca att ctt	682
	His Leu Thr Leu Val Asn His Thr Arg Glu Glu Met Lys Ala Ile Leu	
	115 120 125	
15	cgg gaa tac cta gag ctg gga tta aca aac ctg ttg gcg ctt cga gga	730
	Arg Glu Tyr Leu Glu Leu Gly Leu Thr Asn Leu Leu Ala Leu Arg Gly	
	130 135 140	
20	gat ccg cct gga gac cca tta ggc gat tgg gtg agc acc gat gga gga	778
	Asp Pro Pro Gly Asp Pro Leu Gly Asp Trp Val Ser Thr Asp Gly Gly	
	145 150 155 160	
25	ctg aac tat gcc tct gag ctc atc gat ctt att aag tcc act cct gag	826
	Leu Asn Tyr Ala Ser Glu Leu Ile Asp Leu Ile Lys Ser Thr Pro Glu	
	165 170 175	
30	ttc cgg gaa ttc gac ctc ggt atc gcc tcc ttc ccc gaa ggg cat ttc	874
	Phe Arg Glu Phe Asp Leu Gly Ile Ala Ser Phe Pro Glu Gly His Phe	
	180 185 190	
35	cgg gcg aaa act cta gaa gaa gac acc aaa tac act ctg gcg aag ctg	922
	Arg Ala Lys Thr Leu Glu Glu Asp Thr Lys Tyr Thr Leu Ala Lys Leu	
	195 200 205	
40	cgt gga ggg gca gag tac tcc atc acg cag atg ttc ttt gat gtg gaa	970
	Arg Gly Gly Ala Glu Tyr Ser Ile Thr Gln Met Phe Phe Asp Val Glu	
	210 215 220	
45	gac tac ctg cga ctt cgt gat cgc ctt gtc gct gca gac ccc att cat	1018
	Asp Tyr Leu Arg Leu Arg Asp Arg Leu Val Ala Ala Asp Pro Ile His	
	225 230 235 240	
50	ggt gcg aag cca atc att cct ggc atc atg ccc att acc gag ctg cgg	1066
	Gly Ala Lys Pro Ile Ile Pro Gly Ile Met Pro Ile Thr Glu Leu Arg	
	245 250 255	
55	tct gtg cgt cga cag gtc gaa ctc tct ggt gct caa ttg ccg agc caa	1114
	Ser Val Arg Arg Gln Val Glu Leu Ser Gly Ala Gln Leu Pro Ser Gln	
	260 265 270	
60	cta gaa gaa tca ctt gtt cga gct gca aac ggc aat gaa gaa gcg aac	1162
	Leu Glu Glu Ser Leu Val Arg Ala Ala Asn Gly Asn Glu Glu Ala Asn	
	275 280 285	
65	aaa gac gag atc cgc aag gtg ggc att gaa tat tcc acc aat atg gca	1210
	Lys Asp Glu Ile Arg Lys Val Gly Ile Glu Tyr Ser Thr Asn Met Ala	
	290 295 300	
70	gag cga ctc att gcc gaa ggt gcg gaa gat ctg cac ttc atg acg ctt	1258
	Glu Arg Leu Ile Ala Glu Gly Ala Glu Asp Leu His Phe Met Thr Leu	
	305 310 315 320	

aac ttc acc cgt gca acc caa gaa gtg ttg tac aac ctt ggc atg gcg 1306
 Asn Phe Thr Arg Ala Thr Gln Glu Val Leu Tyr Asn Leu Gly Met Ala
 325 330 335

5 cct gct tgg gga gca gag cac ggc caa gac gcg gtg cgt taagccctct 1355
 Pro Ala Trp Gly Ala Glu His Gly Gln Asp Ala Val Arg
 340 345

10 taggaatcat gaagggggag ggcggtgac aatacggcaa acggccgttg atccccgtca 1415
 aacctaaact gcctgagcaa gtcagtgaag ccgagagagc gatacaggct aaacgcatgg 1475
 ttcgcctcat cgtcgacctc ggggtgtagac aaaatggcaa aagtgttttg tttgtctttt 1535

15 aacagttcat gcatca 1551

<210> 2
 <211> 349
 20 <212> PRT
 <213> Corynebacterium glutamicum

<400> 2
 25 Met Val Glu Val Asn Lys Cys Gln Arg Gln Ser Gln Gln Asn Thr Leu
 1 5 10 15
 Ile Thr Leu Arg Tyr Pro Gly Met Ser Leu Thr Asn Ile Pro Ala Ser
 20 25 30
 30 Ser Gln Trp Ala Ile Ser Asp Val Leu Lys Arg Pro Ser Pro Gly Arg
 35 40 45
 Val Pro Phe Ser Val Glu Phe Met Pro Pro Arg Asp Asp Ala Ala Glu
 50 55 60
 35 Glu Arg Leu Tyr Arg Ala Ala Glu Val Phe His Asp Leu Gly Ala Ser
 65 70 75 80
 40 Phe Val Ser Val Thr Tyr Gly Ala Gly Gly Ser Thr Arg Glu Arg Thr
 85 90 95
 Ser Arg Ile Ala Arg Arg Leu Ala Lys Gln Pro Leu Thr Thr Leu Val
 100 105 110
 45 His Leu Thr Leu Val Asn His Thr Arg Glu Glu Met Lys Ala Ile Leu
 115 120 125
 Arg Glu Tyr Leu Glu Leu Gly Leu Thr Asn Leu Leu Ala Leu Arg Gly
 130 135 140
 50 Asp Pro Pro Gly Asp Pro Leu Gly Asp Trp Val Ser Thr Asp Gly Gly
 145 150 155 160
 55 Leu Asn Tyr Ala Ser Glu Leu Ile Asp Leu Ile Lys Ser Thr Pro Glu
 165 170 175
 Phe Arg Glu Phe Asp Leu Gly Ile Ala Ser Phe Pro Glu Gly His Phe
 180 185 190

Arg Ala Lys Thr Leu Glu Glu Asp Thr Lys Tyr Thr Leu Ala Lys Leu
 195 200 205
 5 Arg Gly Gly Ala Glu Tyr Ser Ile Thr Gln Met Phe Phe Asp Val Glu
 210 215 220
 Asp Tyr Leu Arg Leu Arg Asp Arg Leu Val Ala Ala Asp Pro Ile His
 225 230 235 240
 10 Gly Ala Lys Pro Ile Ile Pro Gly Ile Met Pro Ile Thr Glu Leu Arg
 245 250 255
 Ser Val Arg Arg Gln Val Glu Leu Ser Gly Ala Gln Leu Pro Ser Gln
 260 265 270
 15 Leu Glu Glu Ser Leu Val Arg Ala Ala Asn Gly Asn Glu Glu Ala Asn
 275 280 285
 Lys Asp Glu Ile Arg Lys Val Gly Ile Glu Tyr Ser Thr Asn Met Ala
 290 295 300
 Glu Arg Leu Ile Ala Glu Gly Ala Glu Asp Leu His Phe Met Thr Leu
 305 310 315 320
 25 Asn Phe Thr Arg Ala Thr Gln Glu Val Leu Tyr Asn Leu Gly Met Ala
 325 330 335
 Pro Ala Trp Gly Ala Glu His Gly Gln Asp Ala Val Arg
 340 345
 30
 <210> 3
 <211> 46
 35 <212> DNA
 <213> Artificial sequence
 <220>
 <223> Description of the artificial sequence: Primer
 40 metF-EVP5
 <400> 3
 gatctaggat ccaaaggagg acaaccatgt ccctaacgaa catccc 46
 45
 <210> 4
 <211> 32
 <212> DNA
 <213> Artificial sequence
 50
 <220>
 <223> Description of the artificial sequence: Primer
 metF-EVP3
 55 <400> 4
 gatctactcg agttcttcta gttggctcgg ca 32

What is claimed is:

1. An isolated polynucleotide from coryneform bacteria,
comprising a polynucleotide sequence chosen from the
group consisting of
 - 5 a) polynucleotide which is identical to the extent of
at least 70% to a polynucleotide which codes for a
polypeptide which comprises the amino acid
sequence of SEQ ID No. 2,
 - 10 b) polynucleotide which codes for a polypeptide which
comprises an amino acid sequence which is
identical to the extent of at least 70% to the
amino acid sequence of SEQ ID No. 2,
 - c) polynucleotide which is complementary to the
polynucleotides of a) or b), and
 - 15 d) polynucleotide comprising at least 15 successive
nucleotides of the polynucleotide sequence of a),
b) or c).
2. A polynucleotide as claimed in claim 1, wherein the
polynucleotide is a preferably recombinant DNA which
20 is capable of replication in coryneform bacteria.
3. A polynucleotide as claimed in claim 1, wherein the
polynucleotide is an RNA.
4. A polynucleotide as claimed in claim 2, comprising the
nucleic acid sequence as shown in SEQ ID No. 1.
- 25 5. A DNA as claimed in claim 2 which is capable of
replication, comprising
 - (i) the nucleotide sequence shown in SEQ ID No. 1,
or

- (ii) at least one sequence which corresponds to sequence (i) within the range of the degeneration of the genetic code, or
 - (iii) at least one sequence which hybridizes with the sequence complementary to sequence (i) or (ii), and optionally
 - (iv) sense mutations of neutral function in (i).
6. A polynucleotide sequence as claimed in claim 2, which codes for a polypeptide which comprises the amino acid sequence in SEQ ID No. 2.
7. A coryneform bacterium in which the metF gene is enhanced, in particular over-expressed.
8. A coryneform bacterium serving as the host cell, which contains a vector which carries a polynucleotide as claimed in claim 1.
9. A process for the fermentative preparation of L-amino acids, in particular L-methionine, which comprises carrying out the following steps: .
- a) fermentation of the coryneform bacteria which produce the desired L-amino acid and in which at least the metF gene or nucleotide sequences which code for it are enhanced, in particular over-expressed;
 - b) concentration of the L-amino acid in the medium or in the cells of the bacteria, and
 - c) isolation of the L-amino acid.
10. A process as claimed in claim 9, wherein bacteria in which further genes of the biosynthesis pathway of the desired L-amino acid are additionally enhanced are employed.

11. A process as claimed in claim 9, wherein bacteria in which the metabolic pathways which reduce the formation of the desired L-amino acid are at least partly eliminated are employed.
- 5 12. A process as claimed in claim 9, wherein a strain transformed with a plasmid vector is employed, and the plasmid vector carries the nucleotide sequence which codes for the metF gene.
- 10 13. A process as claimed in claim 9, wherein the expression of the polynucleotide(s) which code(s) for the metF gene is enhanced, in particular over-expressed.
- 15 14. A process as claimed in claim 9, wherein the catalytic properties of the polypeptide (enzyme protein) for which the polynucleotide metF codes are increased.
- 20 15. A process as claimed in claim 9, wherein for the preparation of L-amino acids, in particular L-methionine, coryneform microorganisms in which at the same time one or more of the genes chosen from the group consisting of
- 15.1 the lysC gene which codes for a feed back resistant aspartate kinase,
 - 15.2 the gap gene which codes for glycerolaldehyde 3-phosphate dehydrogenase,
 - 25 15.3 the pgk gene which codes for 3-phosphoglycerate kinase,
 - 15.4 the pyc gene which codes for pyruvate carboxylase,
 - 30 15.5 the tpi gene which codes for triose phosphate isomerase,

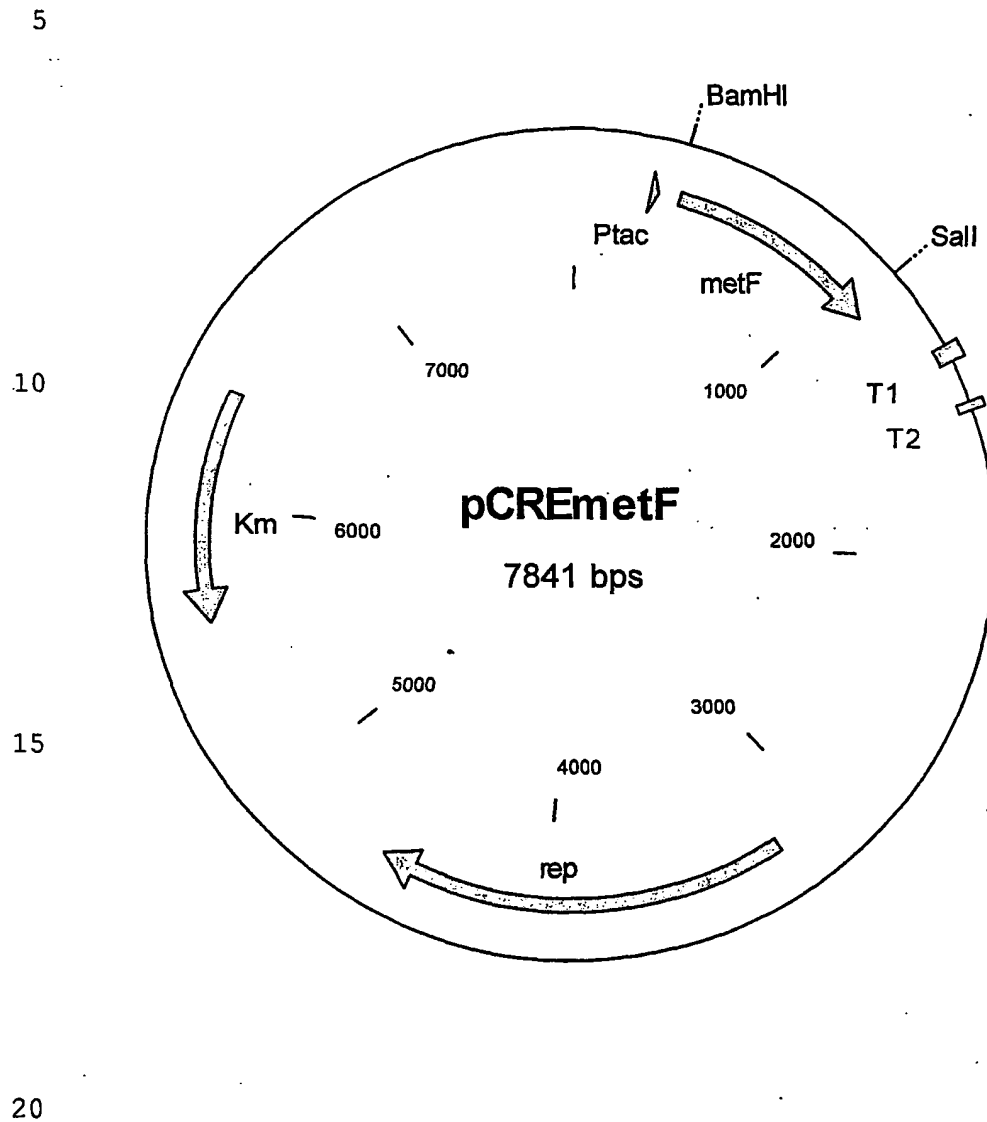
- 15.6 the metA gene which codes for homoserine O-acetyltransferase,
- 15.7 the metB gene which codes for cystathionine gamma-synthase,
- 5 15.8 the aecD gene which codes for cystathionine gamma-lyase,
- 15.9 the glyA gene which codes for serine hydroxymethyltransferase,
- 10 15.10 the metY gene which codes for O-acetylhomoserine sulfhydrylase,
- is or are amplified or over-expressed are fermented.
16. A process as claimed in claim 9, wherein for the preparation of L-amino acids, in particular L-methionine, coryneform microorganisms in which at the same time one or more of the genes chosen from the group consisting of
- 15 16.1 the thrB gene which codes for homoserine kinase,
- 16.2 the ilvA gene which codes for threonine dehydratase,
- 20 16.3 the thrC gene which codes for threonine synthase,
- 16.4 the ddh gene which codes for meso-diaminopimelate D-dehydrogenase,
- 25 16.5 the pck gene which codes for phosphoenolpyruvate carboxykinase,
- 16.6 the pgi gene which codes for glucose 6-phosphate isomerase,

- 16.7 the poxB gene which codes for pyruvate oxidase,
is or are attenuated are fermented.
17. A process as claimed in one or more of claims 9-16,
wherein microorganisms of the species *Corynebacterium*
5 *glutamicum* are employed.
18. A process as claimed in claim 17, wherein the
Corynebacterium glutamicum strain ATCC13032/pCREmetF
is employed.
19. A process for the preparation of an L-methionine-
10 containing animal feedstuffs additive from
fermentation broths, which comprises the steps
- a) culture and fermentation of an L-methionine-
producing microorganism in a fermentation medium;
 - b) 15 removal of water from the L-methionine-containing
fermentation broth (concentration);
 - c) removal of an amount of 0 to 100 wt.% of the
biomass formed during the fermentation; and
 - d) 20 drying of the fermentation broth obtained
according to b) and/or c) to obtain the animal
feedstuffs additive in the desired powder or
granule form.
20. A process as claimed in claim 19, wherein
microorganisms in which further genes of the
biosynthesis pathway of L-methionine are additionally
25 enhanced are employed.
21. A process as claimed in claim 20, wherein
microorganisms in which the metabolic pathways which
reduce the formation of L-methionine are at least
partly eliminated are employed.

22. A process as claimed in claim 20, wherein the expression of the polynucleotide(s) which code(s) for the metF gene is enhanced, in particular over-expressed.
- 5 23. A process as claimed in one or more of claims 19 to 22, wherein microorganisms of the species *Corynebacterium glutamicum* are employed.
24. A process as claimed in claim 23, wherein the *Corynebacterium glutamicum* strain ATCC13032/pCREmetF
10 is employed.
25. A process as claimed in claimed claim 19, wherein one or more of the following steps is or are additionally carried out:
- 15 e) addition of one or more organic substances, including L-methionine and/or D-methionine and/or the racemic mixture D,L-methionine, to the products obtained according to b), c) and/or d);
- 20 f) addition of auxiliary substances chosen from the group consisting of silicas, silicates, stearates, grits and bran to the substances obtained according to b) to e) for stabilization and to increase the storability; or
- 25 g) conversion of the substances obtained according to b) to f) into a form stable to the animal stomach, in particular rumen, by coating with film-forming agents.
26. A process as claimed in claim 19 or 25, wherein a portion of the biomass is removed.
- 30 27. A process as claimed in claim 26, wherein up to 100% of the biomass is removed.

28. A process as claimed in claim 19 or 25, wherein the water content is up to 5 wt.%.
29. A process as claimed in claim 28, wherein the water content is less than 2 wt.%.
- 5 30. A process as claimed in claims 25, 26, 27, 28 or 29, wherein the film-forming agents are metal carbonates, silicas, silicates, alginates, stearates, starches, gums or cellulose ethers.
- 10 31. An animal feedstuffs additive prepared as claimed in claims 19 to 30.
32. An animal feedstuffs additive as claimed in claim 31, which comprises 1 wt.% to 80 wt.% L-methionine, D-methionine, D,L-methionine or a mixture thereof, based on the dry weight of the animal feedstuffs additive.
- 15 33. A process for discovering RNA, cDNA and DNA in order to isolate nucleic acids, or polynucleotides or genes which code for methylene tetrahydrofolate reductase or have a high similarity with the sequence of the methylene tetrahydrofolate reductase gene, which
- 20 comprises employing the polynucleotide sequences as claimed in claim 1, 2, 3 or 4 as hybridization probes.

Figure 1: Plasmid pCREmetF



(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
7 February 2002 (07.02.2002)

PCT

(10) International Publication Number
WO 02/10206 A3

(51) International Patent Classification⁷: C07K 14/34.
C12P 13/08, C12N 1/21, 15/10, 15/63, C12Q 1/68, A23L
1/305, C12N 9/06

(21) International Application Number: PCT/EP01/08224

(22) International Filing Date: 17 July 2001 (17.07.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
100 53 942.4 2 August 2000 (02.08.2000) DE
101 09 686.0 28 February 2001 (28.02.2001) DE

(71) Applicant: DEGUSSA AG [DE/DE]; Bennigsenplatz 1,
40474 Düsseldorf (DE).

(72) Inventors: BATHE, Brigitte; Twieten 1, 33154 Salzkot-
ten (DE). MÖCKEL, Bettina; Benrodestrasse 35, 40597
Düsseldorf (DE). PFEFFERLE, Walter; Jahnstrasse 33,
33790 Halle (Westf.) (DE). HUTHMACHER, Klaus;
Lärchenweg 18, 63584 Gelnhausen (DE). BINDER,
Michael; Kalberkamp 28, 33803 Steinhagen (Westf.)
(DE). GREISSINGER, Dieter; Augasse 1f, 61194
Niddatal (DE). THIERBACH, Georg; Gunststrasse 21,
33613 Bielefeld (DE).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU,
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,
CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH,
GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,
MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK,
SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,
IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF,
CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD,
TG).

Published:

- with international search report
- before the expiration of the time limit for amending the
claims and to be republished in the event of receipt of
amendments

(88) Date of publication of the international search report:
2 May 2002

*For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.*



WO 02/10206 A3

(54) Title: NUCLEOTIDE SEQUENCES WHICH CODE FOR THE METF GENE

(57) Abstract: The invention relates to an isolated polynucleotide comprising a polynucleotide sequence chosen from the group consisting of a) polynucleotide which is identical to the extent of at least 70 % to a polynucleotide which codes for a polypeptide which comprises the amino acid sequence of SEQ ID No. 2, b) polynucleotide which codes for a polypeptide which comprises an amino acid sequence which is identical to the extent of at least 70% to the amino acid sequence of SEQ ID No. 2, c) polynucleotide which is complementary to the polynucleotides of a) or b), and d) polynucleotide comprising at least 15 successive nucleotides of the polynucleotide sequence of a), b) or c), and processes for the fermentative preparation of L-amino acids using coryneform bacteria in which at least the metF gene is present in enhanced form, and the use of the polynucleotide sequences as hybridization probes.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 01/08224

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07K14/34 C12P13/08 C12N1/21 C12N15/10 C12N15/63
C12Q1/68 A23L1/305 C12N9/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K C12N C12P C12Q A23L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, WPI Data, PAJ, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	DATABASE WPI Section Ch, Week 200114 Derwent Publications Ltd., London, GB; Class B04, AN 2001-137957 XP002186601 & WO 01 00843 A (BASF AG), 4 January 2001 (2001-01-04) SEQ ID No 673 and 674 abstract ---	1-8, 32
Y	DATABASE WPI Section Ch, Week 199332 Derwent Publications Ltd., London, GB; Class B05, AN 1993-256072 XP002186165 & KR 9 208 381 B (CHEIL SUGAR & CO LTD), 26 September 1992 (1992-09-26) abstract ---	7-9, 12-14, 17-32
-/--		

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

A document defining the general state of the art which is not considered to be of particular relevance

E earlier document but published on or after the international filing date

L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

G document member of the same patent family

Date of the actual completion of the international search

27 February 2002

Date of mailing of the international search report

20/03/2002

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Seroz, T

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/EP 01/08224

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BLANCO JORGE ET AL: "The folate branch of the methionine biosynthesis pathway in Streptomyces lividans: Disruption of the 5,10-methylenetetrahydrofolate reductase gene leads to methionine auxotrophy." JOURNAL OF BACTERIOLOGY, vol. 180, no. 6, March 1998 (1998-03), pages 1586-1591, XP002191540 ISSN: 0021-9193	1-8,33
Y	page 1586, left-hand column, line 10-23; figure 3	9-32
Y	WO 93 17112 A (GENENCOR INT) 2 September 1993 (1993-09-02) page 1, line 1 -page 2, line 2; examples 1-7	10,11, 15-18, 20,21,23
X	EP 0 358 940 A (DEGUSSA) 21 March 1990 (1990-03-21)	5
Y	page 2, line 16-20 page 8, line 47-49 page 2, line 40-44	7-32
X	EP 0 387 527 A (DEGUSSA) 19 September 1990 (1990-09-19)	5
Y	abstract page 2, line 9-38; table 2	7-32
Y	WO 88 09819 A (MASSACHUSETTS INST TECHNOLOGY) 15 December 1988 (1988-12-15) page 7, line 19 -page 8, line 2 - page 24, line 24 -page 25, line 3; claims 1-4 page 31, line 14 -page 32, line 18; figures 1,2,6	7-32
Y	OLD I G ET AL: "PHYSICAL MAPPING OF THE SCATTERED METHIONINE GENES ON THE ESCHERICHIA COLI CHROMOSOME" JOURNAL OF BACTERIOLOGY, WASHINGTON, DC, US, vol. 175, no. 11, June 1993 (1993-06), pages 3689-3691, XP001037964 ISSN: 0021-9193 abstract table 1	10,11, 15-18, 20-22, 26-32
	--- -/--	

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/EP 01/08224

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>KRAMER R: "Genetic and physiological approaches for the production of amino acids"</p> <p>JOURNAL OF BIOTECHNOLOGY, ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, NL,</p> <p>vol. 45, no. 1,</p> <p>12 February 1996 (1996-02-12), pages 1-21,</p> <p>XP004036833</p> <p>ISSN: 0168-1656</p> <p>-----</p>	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 01/08224

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 0100843	A	04-01-2001	AU 5421300 A	31-01-2001
			WO 0100843 A2	04-01-2001
			AU 5559000 A	31-01-2001
			WO 0100844 A2	04-01-2001
			AU 5836900 A	31-01-2001
			WO 0100804 A2	04-01-2001
			AU 5421600 A	31-01-2001
			WO 0100805 A2	04-01-2001
			AU 5420500 A	31-01-2001
			WO 0100842 A2	04-01-2001
KR 9208381	B	26-09-1992	KR 9208381 B1	26-09-1992
WO 9317112	A	02-09-1993	CA 2130347 A1	02-09-1993
			EP 0630406 A1	28-12-1994
			JP 7503855 T	27-04-1995
			WO 9317112 A1	02-09-1993
EP 0358940	A	21-03-1990	GB 2223754 A	18-04-1990
			DE 68924227 D1	19-10-1995
			DE 68924227 T2	01-02-1996
			EP 0358940 A1	21-03-1990
			JP 2291276 A	03-12-1990
EP 0387527	A	19-09-1990	DE 3908201 A1	27-09-1990
			AT 107699 T	15-07-1994
			DE 59006167 D1	28-07-1994
			EP 0387527 A1	19-09-1990
			ES 2056263 T3	01-10-1994
			JP 3000087 B2	17-01-2000
			JP 3219885 A	27-09-1991
			SK 122890 A3	11-02-1999
WO 8809819	A	15-12-1988	WO 8809819 A2	15-12-1988
			US 5641660 A	24-06-1997